Optimization of a microfluidic assay for the detection of free prostate specific antigen

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***Abstract*—The elevated number of false positives in prostate cancer (PCa) tests have demonstrated the need to design a multiplex assay capable of crossing information from various PCa biomarkers in order to provide a more reliable diagnosis. A miniaturized microfluidic ELISA was optimized in order to detect clinically relevant concentrations (1-4 ng mL^{-1}) of free prostate specific antigen (f-PSA). A microfluidic device comprised of microchannels was microfabricated with polydimethyl siloxane (PDMS) through soft lithography and sealed to a glass slide. The ELISA parameters such as molecules concentration, incubation time, flow rate and blocking methodology were optimized. The detection of concentrations in the ng mL^{-1} range were reached using fluorescence, chemiluminesce and colorimetry methods. Microscope imaging parameters for all three detection methods were optimized. Colorimetry detection was also measured by amorphous silicon (a-Si:H) *p-i-n* photodiodes. Calibration curves based on the detection of f-PSA spiked solutions was constructed. Detection limits were calculated for all the three detection methodologies.**

I. Introduction

PROSTATE cancer (PCa) is the second most common form of cancer worldwide for men arising around 200,000 new cases each year. PCa is also the cancer form that has proliferated the most in the last 20 years. Prostate specific antigen (PSA) is the most popular used serum biomarker for PCa. Normal PSA values have been defined between 1 and 4 ng mL^{-1} in healthy man although these values are to be subject to calibration depending on the historical PSA values of each man. Therefore a method to scan for these small amounts of the PSA in human fluids in a fast, economical and portable way is in need. A further need to reduce current number of false positives crossing of information of clinically relevant biomarkers is become a relevant requisite. Lab-on-chip devices are already taking step in this direction. This methodology tries to implement every step of analytes laboratorial analysis into a single, portable devices. With this point-of-care (PoC) analysis patients can be tested and recieve the test results on the spot allowing for immediate course of action to be taken by the diagnostic entity [1, 2, 3].

Microfluidics provides the tools to execute all these tasks.

In this work a microfluidic device was microfabricated out of Polydimethylsiloxane (PDMS) through soft lithography and sealed onto a glass substrate in which immunoassays were performed for the detection of fPSA biomarker (figure 1). The miniaturization allows for sample and reagent volume reductions and the decrease in the incubation compartment decreases the diffusion distances making the assay many times faster than standard immunoassays. Due to the size of the microchannel laminar flow allows for a tighter control of fluid flow. Microfluidics also allows the use of transparent, biocompatible elastomeres (such as PDMS), easy to fabricated complex fluidic networks and surface functionalization. This becomes possible through the use of soft lithography techniques for the manipulation of the elastomer. The PDMS is a silicon-based organic polymer. It possesses many relevant qualities which justify its common use as substrate in microfluidic structures. It is optically clear, inert, non-toxic and non-flammable and has flexible surface chemistry, low permeability to water and low electrical conductivity. It is also widely used in caulking, lubricating oils, heat-resistant tiles and for the fabrication of contact lenses[4].

Another improvement provided by microfluidics is the possibility to perform an assay using only passive diffusion, removing the need for automated systems which require a power source. This makes the fabrication process even more practical and less expensive. The fact that you have no actuation in a device makes it more reliable in the long term since there is no mechanical wear and tear do to actuation. All of these advantages aligned with the simplicity of a device with no moving parts make the mass production extremely inexpensive when compared to more complex devices. Spotting of antibody solution or other biological molecules is all possible in microfluidics with the use of a nanoplotter. This allows for multiple capture antibodies to be separately patterned on a microchannel [5, 6].

The use of antibodies in different ELISA methodologies is the current standard for monitoring of metabolites in human fluid samples for diagnosis purposes. A miniaturization of a sandwich ELISA assay was optimized for a simple microfluidic devise bearing only a channel with

one inlet and one outlet with the purpose of testing sensitivity of the assay. Antibodies labeled with enzymes such as horseradish peroxidase (HRP) or fluorescein isothiocyanate (FITC) are used to convey signal to the assay. The same labeled antibodies are used in the miniaturized microfluidic ELISA, with associated detection methods for the capturing of the signal. In the case of HPR labeled antibodies a complementary step is necessary in the protocol. HRP conveys a signal through the conversion of a substrate, luminol or 3, 3, 5, 5 -tetramethylbenzidine (TMB). While luminal generates a light emitting reaction, TMB is converted into a light blocking substrate and absorbance of such product is measured. These assays are referred as chemiluminescence or colorimetric assays, respectively. This signal can be detected on the microscope, or by integrated photodiodes aligned with the microchannel in the microfluidic device. The selection of an ELISA for the quantification of biomarkers has demonstrated detection limits in the order of the pg mL-1. Values in this range are suitable for the detection of biological concentrations of PSA although optimization of the immunoassays is required for the specific set of antibodies used for the PSA detection.

PSA detection is not only relevant in terms of absolute concentration but also in terms of ratio of the two different forms of this molecule in human serum. PSA is a glycoprotein enzyme form the kallikrein-related peptidase family secreted by epithelial cells of the prostate gland. PSA has a proteolitic function in its free form and is therefore coupled to a silencing protein to become inactive while in the circulatory serum. The largest amount of PSA is bound to α-1-antichymotrypsin (PSA-ACT) or α-2-macroglobulin (PSA-A2M), forming two complexed forms of PSA. For the purpose of this article the complexed form of PSA will be denominated PSA-ACT from here on. Non-complexed PSA is denominated free PSA (fPSA). Because of these two forms of the molecule a ratio between the two become relevant to define in terms of diagnosis. For this three different antibodies are used in the immunoassay. Free PSA antibody (fPSA), PSA-ACT antibody and equimolar total PSA antibody (emtPSA) are used to establish concentrations and the ratio of each form. The later, emt-PSA antibody, is an innovation to the old total PSA antibody. The issue with this antibody is that the affinity for each form of PSA (free and complexed) would vary according to the ratio of each form in the sample. The emt-PSA antibodies are much more reliable since the affinity for the two forms does not alter due to changes in the ratio. Defining the ratio of these two PSA forms has become relevant for an accurate diagnosis due to the natural variation of basal concentration of this molecule in different men. A correlation between this ration and false positive results has been established. Benign prostate hyperplasia is known to indicate similar results to PCa in terms of fPSA increase in serum. However an analysis to the ratio of free to total PSA has demonstrated significant differences. In studies made to non-prostate cancer and prostate cancer groups has showed that f/t ratios for non-prostate cancer groups was higher (0.18) than for the prostate cancer group (0.088). Having these analysis incorporated to the fPSA amount analysis will help to distinguish between PCa and BPH reducing the false positive results [7, 8].

For the development of a new analytical method, validation and figuring of the detection limits and system sensitivity are required by the regulatory agencies worldwide. There are differences in the methods used for the calculation of different detection limits. These detection limits include various parameters such as the limit of blank (LoB), limit of detection (LoD), limit of quatitation (LoQ) and instrument detection limit (IDL) and method detection limit (MDL). Even when describing the same parameter, some differences are depicted between definitions of a certain limit and even calculation methodologies for each of the different agencies. Three of the most commonly used detection limits were calculated for this project: LoB, LoD and LoQ. The LoB can be defined as the highest apparent signal found on replicates of a blank sample (no analyte). LoD is the lowest quantity of analyte capable of being reliably distinguished from the LoB and at which detection is feasible. LoQ is defined as the lowest concentration at which the analyte can be reliably detected and quantified [9].

II. Methods

A. General overview of experimental procedure

A microfluidic device was microfabricated, containing a straight channel with one inlet and one outlet (figure 1). This device was fabricated using a SU-8 mold to stamp PDMS which was then sealed to a glass slide, previously cleaned. This microfluidic design was used for the immobilization and detection of PSA (prostate specific antigen). The PSA molecule was trapped using a miniaturized ELISA, optimized to be performed inside a microfluidic channel. Flowing of reagents and samples through the channel was made possible with the use of external syringe pumps, controlling the velocity of the

molecules solution inside the channel. The detection was made using fluorescence chemiluminescence and colorimetry. In the case of chemiluminescence and colorimetry, detector antibodies coupled to HRP-enzyme were used and luminal and TMB respectively were flown in the channel and images were captured though a digital camera (Leica DFC300FX) coupled to a Leica microscope (Leica DMLM). In the case of fluorescence, FITC labeled detector antibodies were used, excited by a fluorescence light and images capture by digital camera. Quantification signal intensity was performed by ImageJ software (from NIH).

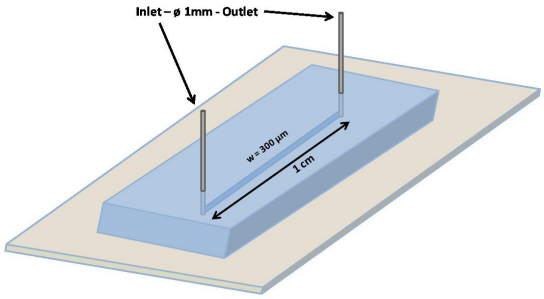

Fig. 1: Microfluidics device basic schematics.

B. *Microfluidic device fabrication*

For the mask fabrication, aluminum was deposited on a clean glass slide by sputtering and then coated with positive photoresist. This slide was then patterned with the computational design using a Direct Write Laser Lithography (DWL) machine. Finally photoresist and aluminum layer were etching thus presenting the desired pattern. The mold is fabricating by coating SU-8 2015 over a clean silicon wafer and spinner to define the height of the SU-8 photoresist. After this the wafer was exposed to UV-light through the previously fabricated mask. Post exposure baking was performed at 95°C for 5 minutes. After etching with propylene glycol monomethyl ether acetate (PGMEA) the mold was baked again for 15 minutes at 130°C. The silicon wafer bearing the SU-8 negative mold was fixed on a petri dish and a mixture of 1:10 of curing agent and base PDMS was poured over the fixed mold. The PDMS was previously mixed and degassed in a vacuum system for 45 minutes. In the end, the petri dish was put in the oven at 70°C during 1h30m in order to cure the PDMS.

C. *Biochemical reactants and sandwich ELISA*

Phosphate saline buffer (PBS) was used for the antibody and antigen dilution and syringe pumps (NE-300, New Era Syringe Pumps) were used to control fluid flow in the microfluidic channel. The sandwich ELISA assay was performed by coating the capture antibody (f-PSA antibody) onto the microchannel surface, and blocking the free space with bovine serum albumin (BSA). After this fPSA antigen solution was flowed through the microchannel followed by the detector antibody (emt-PSA) labeled with HRP or FITC molecule. A washing step was performed in between every step. Capture antibody is specific for fPSA while detector is specific for total PSA (tPSA), this includes fPSA and complexed form PSA. However the epitope specificity is different to avoid competition in the binding site. With this setup a sadwich direct ELISA (figure 2) was performed inside a microfluidics channel for detecting fPSA.

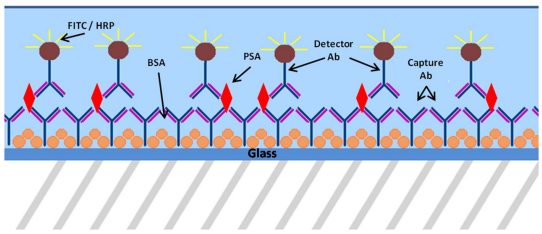

Fig. 2: Schematic representation of a Sandwich ELISA performed inside a microfluidic channel.

D. *Spotting IgG on PDMS*

Using a nanoplotter (GeSim Nanoplotter NpC 2.1) varying concentrations of IgG-FITC solution were microspotted in a microfluidic channel patterned on PDMS which was then sealed to a glass slide. The solutions were dispensed in droplets with controlled over volume (56 pL) and location on a cooled xyz stage. The spots were left for drying inside the nanoplotter with the humidity inside the chamber set at 75%. Fluorescence signal was measured for all of the spots in a fluorescence microscope.

III. Results

In order to optimize the microfluidic assay different parameters were optimized before attempting a fPSA calibration curve with each detection method [11]. Aspects such as surface adsorption, molecule concentration, flow rate of incubation, time of incubation and surface blocking agent were optimized for minimum limit of detection . Detection was made in microscope for the fluorescence and chemiluminescence detection and both in microscope and photodiodes for the colorimetry assay. The data acquisition parameters were also optimized for the detection

of low signals. Spotting experiments were also performed by spotting nano-volumes of antibody solution on a microchannel and measuring the signal intensity and the spot area occupied. Calibrations curves were plotted for the detection of fPSA with all three detection methodologies. Calculations were made for the limit of blank (LoB), limit of detection (LoD) and limit of quantity (LoQ) for all the three detections according to the following formulas:

- LoB = Mean_{blank} + 1,645 * (SD_{blank})
- LoD = ((3,3 * SD_{blank}) - intercept) / slope
- LoQ = ((10 * SD_{blank}) - intercept) / slope

A. Spotting Experiments

Pictures were taken to all spots and fluorescence signal was measured. The fluorescence signal correlates to the concentration of antibody on each spots. Therefore a curve of antibody-FITC concentration versus fluorescence signal was drawn (figure 3). Spotting experiments 1 and 2 results were acquired with 0.2 seconds of exposure. Experiment 3 was imaged with 1 second of exposure. Although there is an inter-experimental exposure difference in this data, is it important to see that the data follows the same trend varying the slope of the increments. This is due to the difference in exposures. By comparing the signal for the 100 µg mL−1 we can easily understand that with a 1 second exposure the results for 500 µg mL−1 spots in the first and second assay would have suffered overexposure.

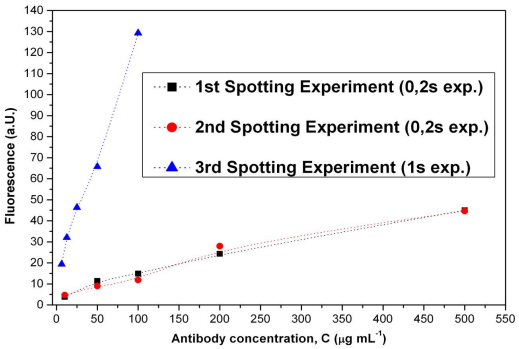

Fig. 3: Fluorescence curves for the 3 spotting experiments. Antibody-FITC concentration used versus Fluorescence signal.

B. Fluorescence signal per molecule

An interesting trend is observed when antibody surface density is plotted against signal per molecule. The spotting experiments allowed for a quantification of the number of molecules on the surface since the experiment required total control over the spotted volume and the droplet area occupied could be easily measured on the microscope with the appropriate calibration. A comparison between the number of pixels measured by ImageJ in each droplet picture was compared to the number of pixels measured for the channel width (300 µm). Knowing the total number of molecules on the spot surface density was calculated. Total amount of signal measured in the spot was divided by the number of molecules present in each spot and plotted against the molecules surface density (figure 4 & 5).

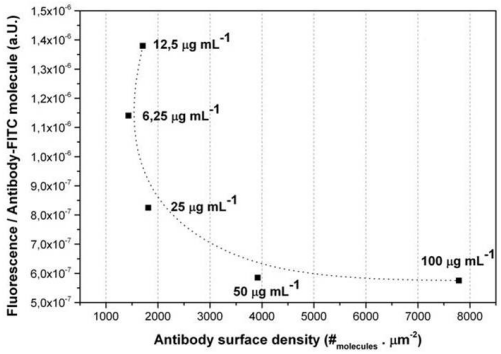

Fig. 4: Fluorescence curves for the first and second spotting experiments. Antibody-FITC concentration used versus Fluorescence signal.

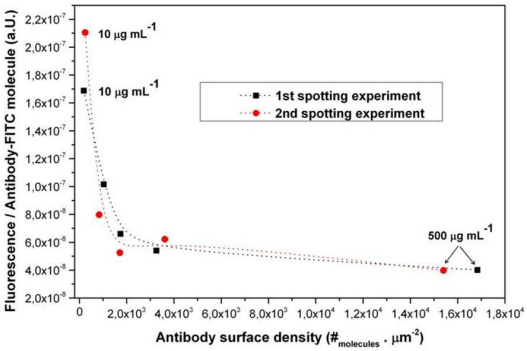

Fig. 5: Fluorescence curves for the third spotting experiment. Antibody-FITC concentration used versus Fluorescence signal.

C. fPSA calibration curves

After optimizing the microfluidic device and the assay detection curves were elaborated in order to evaluate the sensitivity of the different detection methodologies. Fluorescence, chemiluminecence and colorimetry assays were performed using the previously defined parameters for the molecules incubation. For fluorescence a FITC labelled emtPSA antibody was used as detector antibody and for the chemiluminecence and colorimetry the same antibody

was used but labeled with an HRP enzyme instead of FITC molecules. Linear fit for each calibration curve was calculated using Origin 9 analysis tools.

For the ellaboration of the calibration curves, 3 different areas of the channel were measured for each point and a mean and standard deviation calculated for each concentration point. This was done for all experiments measured on the microscope. Fluorescence and chemiluminescence are presented in figure 6 and the colorimetry in figure 7.

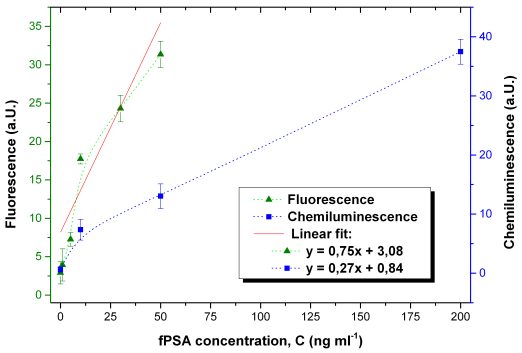

Fig. 6: Fluorescence and chemiluminescence calibration curve using a full sandwich fPSA assay with fluorescence detection method. A linear fit curve was calculated for low concentrations ($\leq$ 50 ng mL^{-1}). The dotted line is just an eye guideline.

The colorimetry curve was measured in two different devices. In the microscope, just as the fluorescence and chemiluminescence assays and using the bottom light source at maximum intensity and fully open condenser, pictures were taken of the transmission plus a control (clear channel) in order to calculate the absorbance of the TMB reaction product. Results presented in figure 7.

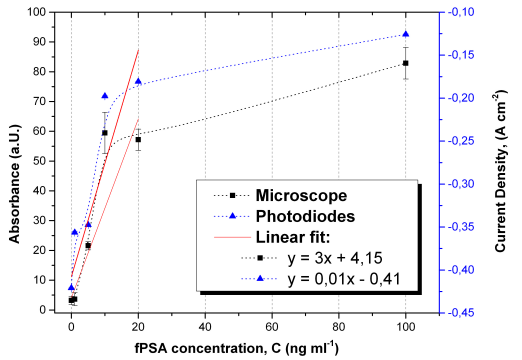

Fig. 7: Colorimetry calibration curve using a full sandwich fPSA assay with fluorescence detection method. A linear fit curve was calculated for low concentrations ($\leq$ 50 ng mL^{-1}). The dotted line is just an eye guideline.

After all the measurements, transmission results were converted to absorbance by normalization with the maximum signal captured through a clear channel. This maximum signal would correspond to the absorbance minimum. Assuming this, all other acquired measurements (including the blank) were subtracted to this maximum signal value. This difference between the two represents the amount of signal absorbed by the TMB precipitate on the channel. The colorimetry assay was also measured in a-Si:H photodiodes. The microfluidic device was aligned on top of a PCB wirebonded to the PDs. This PCB plugged to a picoammeter for current measurements. On top of this assembly, a light source is placed directly on top of the photodiodes, and the amount of light transmitted through each microfluidic channel to the PD is measured in current density (figure 7) [10].

D. Detection limits

Following the previous formulas theselimits of detection were calculated for the three detection methodologies and results are presented in table 1.

TABLE I: Calculated values for LoB, LoD and LoQ for the three detection methods. Colorimetry assay was measured both in microscope (M) and photodiodes (PD). LoD and LoQ units: ng mL^{-1}; LoB units: microscope measurements are in a.U.; Photodiodes in current density (A cm^{-2}).

	Parameters		
	LoB	LoD	LoQ
Fluorescence	5,31	2,30	15,32
Chemiluminescence	1,38	2,44	13,81
Colorimetry (M)	5,41	0,10	3,10
Colorimetry (PD)	-0,421	0,062	0,187

All values used for these calculation came from the results used to build figures 6 and 7. Nevertheless all the data for each calibration curve was performed on the same day and on the same device. Inter-day and inter-device experiments should be performed in order to assess more reliable detection limits.

IV. Discussion

Spotting assay comprised two goals. First to prove the possibility of having a single channel covered with different capture antibodies in clearly defined areas. Also the concentration calibration performed allowed for the characterization of the molecules distribution on the adsorbed surface.

In all three spotting experiments there is a clear tendency to have a diminished value of fluorescence per molecule

when the amount of molecules on the surface (antibody surface density) increases. This is probably due to the fact that molecules packed together will end up muffing the neighbour molecules signal. It would be useful to predict the signal per molecule and use this value to calculate the surface density of molecules in the sandwich assay through the detected fluorescence signal. However an absolute fluorescence value for a single antibody-FITC cannot be defined since this muffing effect will alter the signal detected depending on the same physical magnitude trying to be measured - antibody surface density. Nevertheless for the calculation of a single antibody-FITC signal, having in mind the muffling effect, the use of the lowest concentration possible would be the most correct since there are fewer molecules in the system interfering with the emitted fluorescence.

In table 1 the detection limits calculated for all three detection methods using the microscope are presented. Chemiluminescence presents the highest values for LoD and LoQ, however it presents a low LoB. LoB is not comparable since this is value is presented in a.U. and imaging methodologies differ for the three methodologies. This detection method seems to need the implementation of an amplification methodology that would greatly increase the signals detected. Fluorescence shows LoD and LoQ similar to the chemiluminescence detection. Further optimization of this assay is required mostly to try and decrease the LoB value which would muffle the low concentration signals. By the calculated figures colorimetry seems to be the most sensitive method. However further repetitions of the assay would be necessary to ensure this premise. The standard deviation for the blank using colorimetry detection was in fact the lowest of the three methods. Such a low SD values indicate colorimetry is most likely the most reproducible and reliable detection system.

Colorimetry measured in the microscope presents the highest slope which means that it possesses the largest signal change per analyte quantity. Slope values cannot be compared between the PD and microscope measurements because of the different magnitudes measured (current density and a.U. respectively). Nevertheless a high resolution in the lower concentration values is observable for the colorimetry detection using the PDs. This strengthens the idea of colorimetry as the most reliable method. The fact that the TMB flowing conditions can be adapted to more assertively measure different ranges of concentrations is also a positive argument in favor of colorimetry. The PD results for the colorimetry have little relevance in terms of LoD and LoQ calculation due to the fact that only one measurement was made for each concentration. This measurement is the result of a mean from the values acquired over a minute after the TMB flow was stopped. Therefore the standard deviation calculated is associated with the measuring device error (picoammeter). Another colorimetry curve was measured on the PD but different illumination set ups were used, and therefore these cannot be used for means and SD calculations.

V. Conclusion

All in all none of the detection methods was precise enough to reach the predetermined goal of clinically relevant concentrations for the f-PSA molecule. However colorimetry seems to show great promise. A possible optimization would be the introduction of an amplification system which would greatly benefit the chemiluminescence and colorimetry detections. Using a biotin-streptavidin amplification system this technique could easily become sensitive enough for the measurement of the concentrations of interest (1-4 ng mL^{-1}) mostly due to the very low LoB. This indicates that enzymatic assays reveal more sensitive detection than the fluorescence method. Colorimetry can even benefit from a time based analysis to the increase in absorbance over time during the TMB incubation. A regression can be fit into the increasing absorbance values during the TMB incubation presenting linear fits with different slops which can be then translated into concentrations.

Throughout this work it was demonstrated that biomolecules can be adsorbed on PDMS microchannel with a glass substrate. This allows for an ELISA to be performed in microscale under 45 minutes with minimum molecule consumption. Molecule incubation parameters were optimized for the detection of low concentrations of fPSA. Spotting experiments demonstrated the possibility of sectioning a microfluidics device with different capture antibodies for various PCa biomarkers. This raises the possibility of measuring different analytes and cross-check results of the different molecules for a more accurate diagnosis. An aqueous two-phase system could be implemented for proteins extraction. In an attempt to decrease the assay time, molecules could be premixed with each other (e.g. capture antibody mixed with BSA) in order to decrease incubation time. The measurements using photodiodes show the sensitivity of this biosensor to the enzymatic assay. The integration of PD detection eliminates the need for peripheral equipment, such as the microscope, allowing for the design of a miniature PoC device. All the detection methods were successfully

integrated with the assay. To measure the fluorescence assay on the PD, a special filter (to cut the excitation light) needs to be deposit over the PD. This filter adds an extra number of steps to the microfabrication process of the PD. Also a lateral excitation would probably be required to reduce the scattering of the excitation light [11]. Chemiluminescence presents a further advantage for the PoC device development because it does not require a light source to be integrated into the system. A microfluidic capillary system can be implemented, eliminating the need for external pumps.

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References

[1] D. Huckle. Point-of-care diagnostics: an advancing sector with nontechnical issues. *Expert Rev Mol Diagn*, vol. 8, pp. 679-688, 2008.

[2] M. Desmulliez M.P.Y Mohammed.Lab-on-a-chip based immunosensor principles and technologies for the detection of cardiac biomarkers: a review. *Lab Chip*, vol. 8, pp. 569–595, 2011.

[3] Kadimisetty K. Faria R. Rusling J.F. Sardesai, N.P. A microfluidic electrochemiluminescent device for detecting cancer biomarker proteins. *Anal Bioanal Chem*, vol. 406, pp. 3831âĂŞ3838, 2013.

[4] Kwon K.W. Park M.C. Lee S.H. Kim S.M. Suh-K.Y. Kim, P. Soft lithography for microfluidics: a review. *Expert Rev Mol Diagn*, vol. 8, pp. 679-688, 2008.

[5] D. Huckle. Point-of-care diagnostics: an advancing sector with nontechnical issues. *Biochip Journal*, vol. 2, pp. 1-11, 2008.

[6] Ho C.Wong, I. Surface molecular property modifications for poly(dimethylsiloxane) (pdms) based microfluidic devices. *Microfluid Nanofluidics*, vol. 7, pp. 291-306, 2009.

[7] V. Jarrige. The need for a lower total psa cut-off value with psa assays calibrated to the new who standard., 2007.

[8] Catto-J.W.F. Ward, M.A. and F.C. Hamdy. Prostate-specificantigen: biology, biochemistry and available commercial assays. *Ann.Clin. Biochem.*, vol. 38, pp. 633-651, 2001.

[9] A. C. Pimentel, A. T. Pereira, D. M. F. Prazeres, V. Chu and J. P. Conde. Detection of fluorescently labeled biomolecules immobilised on a detachable substrate using an integrated amorphous silicon photodetector. *Appl. Phys. Lett.*, vol. 94, 2009.

[10] P. Novo, V. Chu and J. P. Conde. Integrated fluorescence detection of labeled biomolecules using a prism-like PDMS microfluidic chip and lateral light excitation. *Lab Chip*, vol. 14, pp. 1991-1995, 2014.